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(54) Title: ADENOVIRUS VECTORS SPECIFIC FOR CELLS EXPRESSING CARCINOEMBRYONIC ANTIGEN AND METHODS OF USE THEREOF

(57) Abstract

Replication–competent adenovirus vectors specific for cells expressing carcinoembryonic antigen (CEA), and methods of use of such viruses are provided. These viruses comprise an adenoviral gene under control of a CEA transcriptional regulatory element (CEA–TRE). The gene can be, for example, a gene required for viral replication or the adenovirus death protein gene (ADP). The viruses can also comprise at least one other adenoviral gene under control of another transcriptional regulatory element specific to cells capable of which allow a CEA–TRE to function, such as a variant of CEA–TRE. By providing for transcriptional initiating regulation dependent upon CEA expression, virus replication can be restricted to target cells which allow a CEA–TRE to function, such as cells expressing CEA, particularly carcinoma cells capable of expressing CEA. An adenovirus of the present invention can further comprise a heterologous gene such as a reporter gene under transcriptional control of a CEA–TRE. The adenovirus vectors can be used to detect and monitor samples for the presence of cells that allow a CEA–TRE to function, as well as to selectively kill malignant cells that allow a CEA–TRE to function.

# ADENOVIRUS VECTORS SPECIFIC FOR CELLS EXPRESSING CARCINOEMBRYONIC ANTIGEN AND METHODS OF USE THEREOF

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/039,763, filed on March 3, 1997.

## STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

(Not applicable)

## TECHNICAL FIELD

This invention relates to cell transfection using adenoviral vectors, especially replication-competent adenoviruses, and methods of their use. More specifically, it relates to cell-specific replication of adenovirus vectors in cells capable of expressing carcinoembryonic antigen (CEA), particularly CEA-associated tumor cells, through use of a carcinoembryonic antigen transcriptional regulatory element (CEA-TRE).

## BACKGROUND OF THE INVENTION

In spite of extensive medical research and numerous advances, cancer remains the second leading cause of death in the United States. Colorectal cancer is the third most common cancer and the second leading cause of cancer deaths. Lung cancer is one of the most refractory of solid tumors because inoperable cases are up to 60% and the 5-year survival is only 13%. In particular, adenocarcinomas, which comprise about one-half of the total lung cancer cases, are mostly chemo-radioresistant. Gastric carcinoma is one of the most prevalent forms of cancers in East Asia, including Japan and Korea. Although extensive surgical operations have been combined with chemotherapy and immunotherapy, the mortality of gastric cancer is still high, due to carcinomatous peritonitis and liver metastasis at advanced stages. Pancreatic cancer is virtually always fatal. Thus, current treatment prospects for many patients with these carcinomas are unsatisfactory, and the prognosis is poor.

(1984) *EMBO J* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; and Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

When used as gene transfer vehicles, adenovirus vectors are often designed to be replication-defective and are thus deliberately engineered to fail to replicate in the target cells of interest. In these vehicles, the early adenovirus gene products E1A and/or E1B are deleted and provided *in trans* by the packaging cell line 293. Graham et al. (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Genetic Virology* 68:937-940. The gene to be transduced is commonly inserted into adenovirus in the deleted E1A and/or E1B region of the virus genome. Bett et al. (1994). Replication-defective adenovirus vectors as vehicles for efficient transduction of genes have been described by, inter alia, Stratford-Perricaudet (1990) *Human Gene Therapy* 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang et al. (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe et al. (1992) *Nat. Gent.* 1:372-378; Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld et al. (1992) *Cell* 68:143-155; Stratford-Perricaudet et al. (1992) *J. Clin. Invest.* 90:626-630; Le Gal Le Salle et al. (1993) *Science* 259:988-990 Mastrangeli et al. (1993) *J. Clin. Invest.* 91:225-234; Ragot et al. (1993) *Nature* 361:647-650; Hayaski et al. (1994) *J. Biol. Chem.* 269:23872-23875; and Bett et al. (1994).

The virtually exclusive focus in the development of adenoviral vectors for gene therapy is use of adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus has been viewed as an undesirable result, largely due to the host immune response. In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert a prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression. There is a need for vector constructs that are capable of eliminating essentially all cancerous cells in a minimum number of administrations before specific immunological response against the vector prevents further treatment.

gene therapy of CEA-positive tumors is described in patent application WO 95/14100. Osaki et al. (1994) describe adenovirus-mediated prodrug gene therapy in which the CEA promoter was used to restrict HSV-tk expression to CEA-producing human lung cancer cells, rendering them sensitive to the nucleoside analog GCV. *Cancer Res.* 54:5258–5261. Similarly, Tanaka et al. (1996) describe using the CEA promoter to drive selectively expression of HSV-tk, which conferred GCV sensitivity to gastric cancer cells. *Cancer Res.* 56:1341-1345. In all of these publications, the adenovirus constructs are replication defective, and the entire focus in experimental approach is using the CEA 5' upstream transcriptional regulatory region(s) to control expression of a non-adenovirus gene. Replication-deficient adenovirus is viewed as a therapeutic gene delivery vehicle, not as an agent per se for effecting selective growth inhibition.

Carcinomas of the gastrointestinal tract are often not curable by standard therapies. Thus, it is critical to develop new therapeutic approaches for these diseases. The present invention addresses this need by providing adenoviral vectors specific for replication in CEA-producing cells.

All publications cited herein are hereby incorporated by reference in their entirety.

## SUMMARY OF THE INVENTION

In one embodiment, the invention provides an adenovirus vector comprising an adenovirus gene under transcriptional control of a carcinoembryonic antigen transcriptional regulatory element (CEA-TRE). A CEA-TRE is capable of mediating gene expression specific to cells capable of expressing CEA or capable of CEA-TRE-mediated transcription. The CEA-TRE can comprise a promoter and/or enhancer from a carcinoembryonic antigen gene, provided that the CEA-TRE is capable of mediating gene expression specific to cells capable of expressing CEA. In one embodiment, a CEA-TRE comprises a promoter from a carcinoembryonic antigen gene. In one embodiment, a CEA-TRE comprises an enhancer from a carcinoembryonic antigen gene. In one embodiment, a CEA-TRE comprises a promoter from a carcinoembryonic antigen gene and an enhancer from a carcinoembryonic antigen gene.

In certain embodiments, the invention provides an adenovirus vector comprising an adenovirus gene under transcriptional control of a CEA-TRE. In one embodiment, a CEA-

gene is a reporter gene. In one embodiment, the heterologous gene is conditionally required for cell survival.

In one embodiment, a composition comprises any adenovirus disclosed herein. In one embodiment, this composition comprises a pharmaceutically acceptable excipient.

In another aspect, the invention provides pharmaceutical compositions comprising an effective amount of an adenovirus vector(s) described herein.

In some embodiments, the invention provides adenovirus vector(s) complexed with a hydrophilic polymer ("masking agent") to create a masked adenovirus. The hydrophilic polymer is attached (covalently or non-covalently) to the capsid proteins of the adenovirus, particularly the hexon and fiber proteins. In preferred embodiments, the adenovirus vectors of the instant invention are complexed with masking agents to create masked adenovirus vectors. In further preferred embodiments, the masking agent is polyethyleneglycol (PEG) covalently linked to an adenovirus vector of the instant invention.

In another aspect, the invention provides kits which contain an adenoviral vector(s) described herein.

In another aspect, the invention provides a host cell transformed with any adenovirus vector(s) described herein.

In another embodiment, a method of treating a CEA-associated tumor in an individual is provided, the method comprising the step of administering to the individual an effective amount of an adenovirus vector in which an adenovirus gene is under transcriptional control of a CEA-TRE. In one embodiment, a CEA-associated tumor comprises cells capable of expressing CEA. In one embodiment, the adenovirus gene is essential for viral replication. In one embodiment, the adenovirus gene is an early gene. In one embodiment, the adenovirus gene is E1A. In one embodiment, the adenovirus gene is E1B. In one embodiment, the adenovirus gene is a late gene. In one embodiment, the adenovirus gene is ADP. In one embodiment, the CEA-TRE comprises an enhancer from a CEA gene. In one embodiment, the CEA-TRE comprises a promoter from a CEA gene. In one embodiment, the CEA-TRE comprises a promoter from a CEA gene and an enhancer from a CEA gene. In one embodiment, the adenovirus further comprises at least one additional adenovirus gene under transcriptional control of at least one additional

In another aspect, methods are provided for conferring selective cytotoxicity on a target cell, said method comprising contacting a cell which allows a CEA-TRE to function with any adenovirus vector(s) described herein, wherein the adenovirus enters the cell.

In one embodiment, an adenovirus is provided which further comprises a heterologous gene under transcriptional control of a CEA-TRE. In one embodiment, the heterologous gene is a reporter gene. In one embodiment, the heterologous gene is conditionally required for cell survival. In one embodiment, a method is provided for detecting cells that allow a CEA-TRE to function in a sample comprising the steps of: contacting a biological sample with an adenovirus vector comprising a heterologous gene under transcriptional control of a CEA-TRE, under conditions suitable for CEA-TRE-mediated gene expression in cells that allow a CEA-TRE to function; and determining if the CEA-TRE mediates gene expression in the biological sample, where CEA-TRE-mediated gene expression is indicative of the presence of cells that allow a CEA-TRE to function.

In one embodiment, a method is provided for modifying the genotype of a target cell, the method comprising contacting a cell that allows a CEA-TRE to function with any adenovirus vector(s) described herein, wherein the vector enters the cell.

In one embodiment, a method for conferring selective cytotoxicity on a target cell is provided, the method comprising contacting a cell that allows a CEA-TRE to function with any adenovirus vector(s) described herein, wherein the vector enters the cell.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a restriction map of the 5' flanking region of the human CEA gene.

Figure 2 depicts the nucleotide sequence of the 5' flanking region of CEA (to about +537).

Figure 3 is a schematic depicting various adenoviral vector constructs as described in Example 1.

Figure 4 is a schematic depiction of an adenoviral vector in which E1A and E1B are under control of a CEA-TRE, with E1A and E1B in opposite orientations.

Figures 5A and B are schematic depictions an adenovirus death protein (ADP) cassette for insertion into Ad. Arrows underneath Fig. 5A indicate positions of primers.

Alternatively, the adenovirus gene under control of a CEA-TRE can be a late replication gene. The adenovirus can optionally comprise at least one other gene such as an adenovirus gene or transgene under control of another TRE which is different from the CEA-TRE. By providing for cell-specific transcription of at least one adenovirus gene required for replication, the invention provides adenovirus vectors that can be used for specific cytotoxic effects due to selective replication. Selective replication is especially useful in the cancer context, in which targeted cell killing is desirable. The adenovirus vectors of this invention are useful for treatment of CEA-associated tumors, such as colorectal carcinomas. The vectors can also be useful for detecting the presence of cells that allow a CEA-TRE to function in, for example, an appropriate biological (such as clinical) sample. Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest in a target cell by using a CEA-TRE.

We have found that the adenovirus vectors of the invention replicate preferentially in cells that allow a CEA-TRE to function, such as cells expressing CEA (i.e., at a significantly higher yield). This replication preference is indicated by comparing the level of replication (i.e., titer) in cells that allow a CEA-TRE to function to the level of replication in cells that do not allow a CEA-TRE to function. The replication preference is even more significant, as the adenovirus vectors of the invention actually replicate at a significantly lower rate in cells that do not allow a CEA-TRE to function than wild-type cells. Comparison of the titer of a cell type that allows a CEA to function to the titer of a cell type that does not allow a CEA-TRE to function provides a key indication that the overall replication preference is enhanced due to depressed replication in cells that do not allow a CEA-TRE to function as well as the replication in cells that allow a CEA-TRE to function. Thus, the invention uses and takes advantage of what has been considered an undesirable aspect of adenoviral vectors, namely, their replication and possibly concomitant immunogenicity. Runaway infection is prevented due to the cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, the inventors note that production of adenovirus proteins can serve to activate and/or stimulate the immune system, either generally and/or specifically toward the target cells producing adenoviral proteins, which can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

enhancer; a CEA promoter and a heterologous (non-CEA) enhancer; a heterologous promoter and a CEA enhancer; and multimers of the foregoing. The promoter and enhancer of a CEA-TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired CEA cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of the protein product of the coding sequence under control of (i.e., operatively linked to) the CEA-TRE. As discussed herein, CEA-TRE can be of varying lengths, and of varying sequence composition. By "transcriptional activation" or "increase in transcription," it is intended that transcription is increased above basal levels in the target cell (i.e., a cell allowing a CEA-TRE to function) by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400-fold to about 500-fold, even more preferably at least about 1000-fold. Basal levels are generally the level of activity (if any) in a cell that does not allow a CEA-TRE to function, or the level of activity (if any) of a reporter construct lacking a CEA-TRE as tested in a cell that allows a CEA-TRE to function.

A "functionally-preserved" variant of a CEA-TRE is a CEA-TRE which differs from another CEA-TRE, but still retains the ability to increase transcription of an operably linked polynucleotide, especially cell-specific transcription activity. The difference in a CEA-TRE can be due to differences in linear sequence, arising from, for example, single or multiple base mutation(s), addition(s), deletion(s), insertion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a CEA-TRE.

As used herein, "expression in cells that allow a CEA-TRE to function", "expression specific to cells capable of expressing CEA," and the like indicate gene expression which occurs primarily in cells containing all the transcriptional factor(s) and/or co-factor(s) needed to mediate transcription from a CEA-TRE, but to a lesser degree in other cells. These terms (as well as the terms "transcriptional activation" or an "increase in transcription") indicate that this gene expression is at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more

this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothiate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) J. Immunol. 141: 2084-9; Latimer et al. (1995) *Mol. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support.

One of skill in the art would recognize that point mutations and deletions can be made to a CEA-TRE disclosed herein without altering the ability of the sequence to increase transcription. Preferably, the polynucleotide of the present invention is DNA. As

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce, or proliferate. This term is well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

A "heterologous gene" or "transgene" is any gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell, prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

A "heterologous" promoter or enhancer is one which is not associated with or derived from a CEA 5' flanking sequence. Examples of a heterologous promoter or enhancer are the albumin promoter or enhancer and other viral promoters and enhancers, such as from SV40.

An "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of any vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or of total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected *in vivo* with a vector of this invention.

A "target cell" is any cell that allows a CEA-TRE to function. Preferably, a target cell is a mammalian cell, preferably a cell that allows a CEA-TRE to function, more preferably a human cell that allows a CEA-TRE to function, such as a cell that expresses CEA.

As used herein, "neoplastic cells" and "neoplasia" refers to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype

sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread (i.e., metastasis) of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

A "masked adenovirus" is an adenovirus which has been complexed with a hydrophilic polymer ("masking agent"). The adenovirus may be any adenovirus, including naturally occurring isolates of adenovirus or engineered adenovirus vectors such as those disclosed in the instant application. Masking agents are preferably of low immunogenicity. Examples of acceptable hydrophilic polymers include: polyethylene/polypropylene copolymers, polyacrylic acid analogues, sugar polymers such as cellulose, polyformaladehyde, poly(N-vinylpyrollidone), polyethylene glycol (PEG), and the like. The hydrophilic polymer may be complexed by covalent or non-covalent attachment to the capsid proteins of the virus, particularly the hexon and fiber capsid proteins. A preferred hydrophilic polymer is PEG, and a preferred masked adenovirus is PEG covalently linked to adenovirus ("covalently pegylated adenovirus").

"Palliating" a disease means that the extent and/or undesirable clinical manifestations of a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not administering adenoviral vectors of the present invention.

***Adenoviral vectors having replication specificity for cells that allow a CEA-TRE to function***

The present invention also provides adenoviral vector constructs which comprise an adenoviral gene under transcriptional control of a CEA-TRE. Preferably, the adenoviral gene is one that contributes to cytotoxicity (whether directly and/or indirectly), more preferably one that contributes to or causes cell death and even more preferably, the

selective expression of a reporter construct in CEA-producing LoVo and SW1463 cells. Richards et al. (1995) also localized the promoter to about -90 and about +69 relative to the transcriptional start site, with region about -41 to about -18 as essential for expression. WO95/14100 describes a series of 5' flanking CEA fragments which confer cell-specific activity, which are herein defined as enhancers or CEA-TRE enhancers, such as about -299 to about +69; about -90 to about +69; about -14.5 to about -10.6; about -13.6 to about -10.6, about -6.1 to about -3.8. The following combinations of regions are examples of CEA-TREs (numbering according to Figure 2): (a) about -299 to about +69; (b) about -1664 to about +69; (c) about -14462 to about -10691 plus about -299 to about +69; (d) about -89 to about -40 plus about -90 to about +69; (e) [3 x (about -89 to about -40)] plus about -90 to about +69; (f) about -3919 to about -6071 plus about -299 to about +69; (g) about -6071 to about -3919 plus about -299 to about +69; (h) about -13579 to about -10691 plus about -89 to about -40 plus about -90 to about +69; (i) about -10691 to about -13579 plus about -89 to about -40 plus about -90 to about +69; (j) about -14500 to about -10600 plus about -6100 to about -3900 plus about -299 to about +69; (k) about -13600 to about -10600 plus about -6100 to about -3900 plus about -299 to about +69; (l) about -3900 to about -6100 plus [4 x (about -90 to about +69)]; (m) about -13600 to about -10600 plus [4 x (about -90 to about +69)]. Thus, any of the above CEA-TREs may be used in the invention as long as requisite desired functionality is displayed in the adenoviral vector.

In one embodiment, the CEA-TRE comprises an approximately 0.5 kb promoter (about -402 to about +69, SEQ ID NO:1), which is specific for CEA-producing cells. Accordingly, the invention also includes an adenovirus vector in which the CEA-TRE comprises SEQ ID NO:1.

A CEA-TRE can also comprise multimers. For example, a CEA-TRE can comprise a tandem series of at least two, at least three, at least four, or at least five CEA promoter fragments (such as the 440-bp fragment described by Schrewe et al.). Alternatively, a CEA-TRE could have one or more CEA promoters along with one or more CEA enhancers.

A CEA-TRE of the present invention may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) can assist in shutting off transcription (and thus replication) in non-permissive (i.e., non-CEA-producing) cells. Thus, presence of a silencer can confer enhanced cell-specific replication by more effectively preventing

additional CEA-TRE(s) differ from the first. In this way, for example, the possibility of homologous recombination with concomitant loss of intervening sequences can be avoided. The first and additional CEA-TREs can, for example, differ in sequence in essential or non-essential regions. For example, the first CEA-TRE could comprise a CEA enhancer and a non-CEA promoter; an additional CEA-TRE could comprise a non-CEA enhancer and a CEA promoter. Alternatively, the essential portions of the promoter and/or enhancer could be identical in both, with the intervening non-essential regions different. In one embodiment, where one CEA-TRE mediates transcription of one gene, and at least one other CEA-TRE mediates transcription of another gene, the orientation of the genes is divergent or convergent, rather than tandem. In this way, any recombination between the CEA-TREs is unlikely to result to deletion of the intervening sequences.

In some embodiments, the invention provides adenoviral vectors which comprise an additional adenovirus gene under transcriptional control of a second CEA-TRE. Examples of an additional adenovirus gene under transcription control is ADP (discussed above) and genes necessary for replication, such as early genes. For example, an adenoviral vector can be constructed such that a first CEA-TRE regulates transcription of one early gene, such as E1A or E1B, and a second CEA-TRE regulates transcription of another early gene. These multiple constructs may be more desirable in that they provide more than one source of cell specificity with respect to replication.

For example, a CEA-TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A, and at least one other CEA-TRE with a different sequence can be introduced immediately upstream of and operably linked to another early gene such as E1B.

Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having a single or multiple adenovirus gene(s) under control of a CEA-TRE, a reporter gene(s) are also under control of a CEA-TRE.

For example, a CEA-TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A or E1B, and this construct may also contain at least one other CEA-TRE driving expression of a reporter gene. The reporter gene can encode a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the *lacZ* gene), luciferase, alkaline

more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400-to about 500-fold, even more preferably at least about 1000-fold. Comparisons between or among various CEA-TREs can be assessed by measuring and comparing levels of expression within a single CEA-producing cell line. It is understood that absolute transcriptional activity of a CEA-TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the CEA-TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

Alternatively a putative CEA-TRE can be assessed for its ability to confer adenoviral replication preference for cells that allow a CEA-TRE to function. For this assay, constructs containing an adenovirus gene essential to replication operatively linked to a putative CEA-TRE are transfected into cells that express CEA. Viral replication in those cells is compared, for example, to viral replication by wild type adenovirus in those cells and/or viral replication by the construct in cells not expressing CEA. A more detailed description of this kind of assay is in Example 2.

It is understood that, to practice this invention, it is not necessary to use CEA-TREs having maximum activity, or having minimum size. The requisite degree of activity is determined, inter alia, by the anticipated use and desired result. For example, if an adenoviral vector of the invention is used to monitor cells for CEA-producing activity, it is possible that less than a maximal degree of responsiveness by a CEA-TRE will suffice to qualitatively indicate the presence of such cells. Similarly, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be sufficient for the desired result, if, for example, the CEA-producing cells are not especially virulent and/or the extent of disease is relatively confined.

The size of CEA-TREs will be determined in part by the capacity of the adenoviral vector, which in turn depends upon the contemplated form of the vector (see below).

differential of at least 2-fold is preferred; more preferably, at least 5-fold; more preferably, at least 10-fold; more preferably, at least 50-fold; even more preferably, at least 100-fold; still more preferably, at least 200-fold; still more preferably, at least about 400-fold to about 500-fold; even more preferably, at least 1000-fold. The acceptable differential can be determined empirically (using, for example, Northern assays or other assays known in the art or assays described in the Example section) and will depend upon the anticipated use of the adenoviral vector and/or the desired result.

Suitable target cells are any cell type that allows a CEA-TRE to function, such as cells that express, or produce, or are capable of expressing or producing CEA. Especially preferred are CEA-associated tumor (carcinoma) cells including, but not limited to, colorectal, gastric, pancreatic, lung, and breast cells and any metastases of the foregoing. CEA production can be measured using assays standard in the art, such as RIA, ELISA or Western blots (immunoassays) to determine levels of CEA protein production or Northern blots to determine levels of CEA mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to transcriptionally activate a CEA-TRE (i.e., allow a CEA-TRE to function).

Any of the various serotypes of adenovirus can be used, such as Ad2, Ad5, Ad12, and Ad40. For purposes of illustration, the serotype Adenovirus 5 (Ad5) is exemplified herein.

In some embodiments, a CEA-TRE is used with an adenovirus gene that is essential for propagation, so that replication competence is preferentially achievable in a target cell that allow a CEA-TRE to function. Preferably, the gene is an early gene, such as E1A, E1B, E2, or E4. (E3 is not essential for viral replication.) More preferably, the early gene under CEA-TRE control is E1A and/or E1B. More than one early gene can be placed under control of a CEA-TRE. Example 1 (Figure 3) provides a more detailed description of such constructs.

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a trans-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* 651:175–208; Flint (1986)

of the stop codon for the above mentioned 33 kDa protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a CEA-TRE having SpeI ends into the SpeI site in the l-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow AR-restricted expression of E2 transcripts.

The E4 gene produces a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames (ORFs) 3 and 6 can both perform these functions by binding the 55-kDa protein from E1B and heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55-kDa protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than $10^{-6}$ that of wild type virus. To further restrict viral replication to CEA-producing cells, E4 ORFs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a vector with sequences in which the E1B region is regulated by a CEA-TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on an CEA-TRE driving E1B.

The major late genes relevant to the subject invention are L1, L2, L3, L4 and L5 which encode proteins of the Ad5 virus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at about +5986 to about +6048.

In one embodiment, an early gene such as E1A or E1B gene is under control of a CEA-TRE.

In one embodiment, E1A and E1B are under control of one or more CEA-TREs by making the following construct. A fragment containing the coding region of E1A through the E1B promoter is excised from the Ad genome and reinserted in opposite orientation (Figure 4). In this configuration, the E1A and E1B promoters are next to each other, followed by E1A in opposite orientation (so that neither the E1A or E1B promoters are operatively linked to E1A), followed by E1B in opposite orientation with respect to E1A. An CEA-TRE(s) can be inserted between E1A and E1B coding regions, (which are in opposite orientation), so that these regions ore under control of the TRE(s). Appropriate

such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly, genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. awsin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN-α, -β, -γ, TNF-α, -β, TGF-α, -β, NGF, and the like. The positive effector genes could be used in an early phase, followed by cytotoxic activity due to replication.

In some embodiments, the adenovirus death protein (ADP), encoded within the E3 region, is maintained (i.e. contained) in the adenovirus vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

Accordingly, the invention provides a adenoviral vectors that includes a polynucleotide sequence encoding an ADP. A DNA sequence encoding an ADP and the amino acid sequence of an ADP are depicted in SEQ ID NO:19 and SEQ ID NO:20, respectively. Briefly, an ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which ADP has been more fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and ligated to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) can then be introduced into the adenoviral genome, for example, in the E3 region (where the ADP coding sequence will be driven by the MLP or the E3 promoter). The ADP coding sequence could also be inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the ADP coding sequence could be operably linked to a heterologous promoter (with or without enhancer(s)), including, but not limited to, another viral promoter, a tissue specific promoter such as AFP (alpha fetoprotein), carcinoembryonic antigen (CEA), mucin, and rat probasin. Example 5 provides a description of an ADP construct in which the coding sequence for ADP was inserted into the E3 region of Ad5.

If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus(or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art, such as calcium phosphate precipitation, electroporation, direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in packaged adenoviruses, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about $10^4$ to about $10^{14}$. The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to about 1000 μg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple, simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

In some embodiments, an adenovirus vector(s) is complexed to a hydrophilic polymer to create a masked adenovirus. The hydrophilic polymer is attached (covalently or non-covalently) to the capsid proteins of the adenovirus, particularly the hexon and fiber proteins. In preferred embodiments, the adenovirus vectors of the instant invention a complexed with masking agents to create masked adenovirus vectors. Masked adenoviruses are advantageous due to (a) the masking of the adenovirus surface to adenovirus neutralizing antibodies or opsinins which are in circulation, and (b) increasing systemic circulation time of adenovirus particles by reduction of non-specific clearance mechanism in the body (i.e., macrophages, etc.). In the *in vivo* context, the systemic delivery of a masked adenovirus results in a longer circulation of viral particles, less

like. Quaternary amine groups are useful as positively charged moieties for electrostatic, non-covalent attachment of masking agents to adenovirus. Masking agents containing or modified or synthesized to contain hydrophobic groups, such as lipids (*e.g.*, phosphatidylethanolamine and the like) and other hydrophobic groups (such as phenyl groups or long alkyl chains), can be complexed to adenoviral vectors by hydrophobic interaction with stable hydrophobic regions on the virus. Affinity masking agents can be made using any small molecule, peptide or protein which binds to adenovirus. The affinity and hydrophobic moieties may be attached to the masking agent by any method known in the art, preferably by chemical crosslinking with a chemical crosslinker.

If the masking agent is covalently attached, a chemical crosslinker is preferably used to covalently bond the masking agent to the adenovirus. The crosslinker may be any crosslinker capable of creating a covalent linkage or bridge between the masking agent and the adenovirus. Direct crosslinking, in which the adenovirus, masking agent and a separate crosslinker molecule are reacted, may be employed to created covalently masked adenovirus, using any chemical crosslinker known in the art which will create crosslinks between the masking agent and protein. Either the masking agent or the adenovirus may be modified prior to the crosslinking reaction, so that the chemical crosslinker will react with the two molecules (*e.g.*, the masking agent may be modified to add amine groups, allowing it to be crosslinked to the adenovirus by crosslinking agents which react with amines).

Preferably, either the masking agent or the adenovirus is first activated by reaction with a crosslinking agent. Unreacted crosslinker is then removed from the masking agent or adenovirus. The activation reaction preferably results in one or two molecules of crosslinking agent per molecule of masking agent, more preferably a single molecule of crosslinking agent per molecule of masking agent. The activated masking agent or adenovirus is then mixed with adenovirus (if the masking agent is activated) or masking agent (if the adenovirus is activated) under the appropriate reaction conditions to form masked adenovirus. Preferably, the masking agent is activated, then reacted with adenovirus.

The preferred masking agent is PEG. Preferred activated PEGs include, but are not limited to: nucleophilic crosslinking PEGs such as end terminal amine PEG, PEG amino acid esters, PEG hydrazine hydrochloride, thiol PEGs, and the like; carboxyl PEGs

useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Preferably, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing (1990). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of CEA-producing cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

*Preparation of the adenovirus vectors of the invention*

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, a CEA-TRE is inserted 5' to the adenoviral gene of interest, preferably an adenoviral replication gene, more preferably one or more early replication genes (although late gene(s) can be used). A CEA-TRE can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for a CEA-TRE. Accordingly, convenient

limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a 30 sequence change resulting in a unique restriction site, one can provide for insertion of CEA-TRE at that site. Example 1 provides a more detailed description of an adenoviral vector in which E1A is under CEA-TRE control.

A similar strategy may also be used for insertion of a CEA-TRE element to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a CEA-TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with the cell-specific response element regulating E1A, as the template for introducing a CEA-TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. Example 1 provides a more detailed description of how such constructs can be prepared.

Similarly, a CEA-TRE can be inserted upstream of the E2 gene to make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Imm.* (1995) 199(part 3):177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a CEA-TRE having SpeI ends into the SpeI site in the 1-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow CEA-restricted expression of E2 transcripts.

chromatography, electrophoretic methods, or filtration methods such as dialysis, diafiltration or ultrafiltration.

***Methods using the adenovirus vectors of the invention***

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

In one embodiment, methods are provided for conferring selective cytotoxicity in cells that allow a CEA-TRE to function, preferably cells expressing CEA, comprising contacting such cells with an adenovirus vector described herein. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, $^{3}$H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow an CEA-TRE to function, preferably mammalian cells expressing CEA. These methods entail combining an adenovirus vector with the cells, whereby said adenovirus is propagated.

Another embodiment provides methods for killing cells that allow a CEA-TRE to function in a mixture of cells, comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of normal cells and cancerous cells that allow a CEA-TRE to function, and can be an *in vivo* mixture or *in vitro* mixture.

The invention also includes methods for detecting cells which allow a CEA-TRE to function, such as those capable of expressing CEA in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. In one method, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. Alternatively, the sample can be contacted with an adenovirus in which a reporter gene is under control of a CEA-TRE. When such an adenovirus is introduced into a biological sample, expression of the reporter gene indicates the presence of cells that allow a CEA-TRE to function. Alternatively, an adenovirus can be constructed in which a gene conditionally required for cell survival is placed under

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic toward cells that allow a CEA-TRE to function. Tumor cell markers include, but are not limited to, PSA, hK2, and carcinoembryonic antigen (CEA). Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. Treatment using an adenoviral vector(s) is indicated in individuals with CEA-associated tumors as described above, such as hepatocellular carcinoma. Also indicated are individuals who are considered to be at risk for developing CEA-associated tumors (including single cells), such as those who have had disease which has been resected and those who have had a family history of CEA-associated tumors. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) in a pharmaceutically acceptable excipient is administered. Pharmaceutical compositions are described above.

The amount of adenoviral vector(s)to be administered will depend on several factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular CEA-TRE employed, and the particular vector construct (i.e., which adenovirus gene(s) is under CEA-TRE control).

If administered as a packaged adenovirus, from about $10^4$ to about $10^{14}$, preferably from about $10^4$ to about $10^{12}$, more preferably from about $10^4$ to about $10^{10}$. If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01 μg to about 100 μg can be administered, preferably 0.1 μg to about 500 μg, more preferably about 0.5 μg to about 200 μg. More than one adenoviral vector can be administered, either simultaneously or sequentially. Administrations are typically given periodically, while monitoring any

## EXAMPLES

### Example 1

**Adenovirus vectors containing a CEA-TRE driving transcription of E1A and/or E1B**

**1. A. The carcinoembryonic antigen transcriptional response element (CEA-TRE)**

The transcriptional response element of the carcinoembryonic antigen (CEA-TRE), about -402 to about +69 bp relative to the transcriptional start (SEQ ID NO:1), was amplified by polymerase chain reaction (PCR) from human genomic DNA using primers:

5' ATT ACC GGT AGC CAC CAC CCA GTG AG 3' (39.174B, upper primer) (SEQ ID NO:9)

and

5' TAG ACC GGT GCT TGA GTT CCA GGA AC 3' (39.174D) (SEQ ID NO:10).

A unique restriction site AgeI was introduced by the primer pair at the ends of the PCR amplified product.

The CEA-TRE PCR fragment was ligated into pGEM-T vector (Promega) which had been linearized with EcoRV. The ligation mixture was transformed into *E. coli* DH5α cells. The desired clone, carrying a CEA-TRE fragment, was obtained and designated CN265.

**1. B. Construction of CEA-TRE adenoviruses comprising one or two adenovirus genes under transcriptional control of CEA-TRE**

Three replication-competent, CEA cell-specific adenoviruses were produced:

CN741, which contains a CEA-TRE driving the expression of the E1A gene;

CN742, which contains two CEA-TREs driving expression of the E1A and E1B genes; and

CN743, which contains a CEA-TRE driving E1B expression.

The viruses were generated by homologous recombination in 293 cells and cloned by plaque purification. The structure of the genomic DNA was analyzed by PCR and

contains an AgeI site at Ad nt 547. This DNA segment was used to replace the corresponding segment of pXC.1 to create CN95.

An EagI site was created upstream of the E1B start site by inserting a G residue at Ad5 nt 1682 by oligonucleotide directed mutagenesis as above. To simplify insertion of a CEA-TRE in the EagI site, the endogenous EagI site in CN95 was removed by digestion with EagI, treatment with mung bean nuclease, and re-ligation to construct CN114. The following primers were used to amplify the segment between 1682 and the KpnI site at Ad5 nt 2048:

15.133A, 5'-TCGTCTTCAAGAATTCTCA (SEQ ID NO:2), containing an EcoRI site, and

9.4, 5'-GCCCACGGCCGCATTATATAC (SEQ ID NO:6), containing an EagI site

9.3, 5'-GTATATAATGCGGCCGTGGGC (SEQ ID NO:7), containing an extra G as well as an EagI site, and

24.020, 5'-CCAGAAAATCCAGCAGGTACC (SEQ ID NO:8), containing a KpnI site.

Co-amplification of the two segments with primers 15.133A and 24.020 yielded a fragment with an EagI site at Ad5 nt 1682 which was used to replace the corresponding EcoRI/KpnI site in pXC.1 to construct CN124.

A CEA-TRE fragment excised from CN265 (see above) by digestion with PinAI was ligated into similarly digested CN124 (which contains the left hand end of the adenovirus) to generate CN266. CN266 is a vector comprising the left-hand portion of adenovirus, in which a CEA-TRE is inserted upstream of and controls expression of E1A.

The full-length CEA-E1A virus, designated CN741, was constructed by homologous recombination of CN266 and BHG11, which contains the right hand side of Adenovirus 5. Briefly, the plasmid CN266 was digested with PvuI; BHG11, with ClaI. Equivalent amounts (5 µg) of each linearly cut plasmid were transfected into 293 cells with a 4-fold excess of cationic liposomes such as Lipofectin DOTAP/DOPE (1:1). 293 is a human embryonic kidney cell line which efficiently expresses the E1A and E1B genes of Ad5 and exhibits a high transfection efficiency with adenovirus DNA. 8 days after infection, viral plaques were observed on the cell monolayer; cells/viruses were harvested, freeze-thawed 3x, centrifuged to pellet the cellular debris, and the supernatant collected. CN741 was plaque-purified three times.

These assays confirmed the identity of CN741, the full-length adenovirus in which a CEA-TRE controls E1A expression.

**1. B. 2. CEA-TRE-driven E1B adenovirus plasmid (CN743)**

Briefly, a CEA-TRE fragment was inserted into CN124 (described above) to generate CN290, which comprises the left-hand end of adenovirus with a CEA-TRE controlling expressing of the adenovirus E1B gene. CN290 was recombined with a plasmid carrying the right-hand portion of adenovirus to generate CN743, which is a full-length adenovirus in which CEA-TRE controls expression of adenovirus gene E1B.

In more detail, the CEA-TRE was obtained as an EagI fragment from CN284 (described below). This fragment was isolated by gel electrophoresis and inserted into CN124, similarly cut with EagI. CN124, also described above, contains the left-hand portion of Adenovirus 5, with an artificial EagI site upstream of the E1B start site. The resulting clone, designated CN290, has a CEA-TRE inserted upstream of the E1B in a left-hand portion of adenovirus. The identity of CN290 was confirmed by restriction digest (ScaI: 2937 and 7406 bp; SmaI: 180, 783, 2628, and 6752 bp).

CN743 was generated by homologous recombination by co-transfecting 293 cells, which produces E1B, with CN290 and BHG11, which contains the wt right hand portion of Ad5. Thus, CN743 is a full-length adenoviral genome in which gene E1B is under control of a CEA-TRE.

**1. C. Construction of adenovirus vectors in which expression of two adenovirus genes are each controlled by a CEA-TRE (CN742)**

Briefly, a CEA-TRE fragment was inserted upstream of the E1B gene in construct CN266, which already had a CEA-TRE fragment inserted upstream of E1A. The resulting plasmid was designated CN285 and contained a left-hand portion of adenovirus with separate copies of a CEA-TRE driving expression of E1A and E1B. CN285 was recombined with a right-hand portion of adenovirus to generate CN742, which is a full-length adenovirus in which expression of both E1A and E1B is controlled by CEA-TRE.

In more detail, CN285 was constructed by amplifying the CEA-TRE inserted into the E1A region (e.g., CN266) by PCR using primers:

Growth selectivity of CN741, CN742 and/or CN743 (full-length adenoviruses in which one or two early genes is under control of a CEA-TRE) is analyzed in plaque assays in which a single infectious particle produces a visible plaque by multiple rounds of infection and replication. Virus stocks are diluted to equal pfu/ml, then used to infect monolayers of cells for 1 hour. Comparison of normalized titres in cells that allow a CEA-TRE to function and cells that do not allow a CEA-TRE to function indicates replication preference. Cells chosen for this study are cells that allow a CEA-TRE to function, such as NCIH508, LoVo, SW1463, MKN1, MKN28, MKN45 and cells that do not allow such function, such as HuH7 or HeLa. The inoculum is then removed and the cells are overlayed with semisolid agar containing medium and incubated at 37°C for one week. Plaques in the monolayer are then counted and titers of infectious virus on the various cells are calculated. The data are normalized to the titer of CN702 (wild type) on 293 cells.

Full-length adenovirus CN741, in which transcription of E1A is under control of CEA-TRE, was tested in this way. Clone 46.130.8.3 was used, and CN702 (wt adenovirus) was a control. Plaques observed on cell lines were normalized to infectivity on control 293 Cells. The ratio of normalized plaques of CN741 and CN702 were compared to evaluate plaque preference in cell types. Table 2 depicts the plaque assay results. Cells examined were 293 (CEA-deficient), LoVo (CEA-producing), OVCAR (CEA-deficient), HBL100 (CEA-deficient), and HepG2 (CEA-producing). We have found that OVCAR and HBL100 cells do not express levels of CEA detectable by ELISA, using a standard protocol with a kit purchased from Genzyme. However, while we also found that HepG2 cell do not produce CEA detectable in the ELISA test, Zhai et al. [(1990) *Gastroenter.* 98:470-7] showed that HepG2 cells *do* produce CEA, as detectable by the PAP and avidin-biotin technique.

*Table 2*

*Plaque assay results of CN741 (CEA-E1A) on human cell lines*

| Cell Line | Normalized Plaques CN702 (wt) | Normalized Plaques CN741 (CEA-E1A) | Ratio of CN741/CN702 |
|---|---|---|---|
| 293 | 1.0 | 1.0 | 1.0 |

An initial indicator of the feasibility is to test the vector(s) for cytotoxic activity against LoVo tumor xenografts grown subcutaneously in Balb/c nu/nu mice. Mice are given s.c. injections with 1 X $10^7$ LoVo carcinoma cells in PBS. Tumor cells can be tested for CEA production by assaying for CEA in serum using standard assays (for example, ELISA).

For this experiment, test adenovirus vectors are introduced into the mice either by direct intratumoral, intravenous, or intraperitoneal injection of approximately $10^8$ pfu of virus (if administered as a packaged virus) in 0.1 ml PBS and 10% glycerol or intravenously via the tail vein. If administered as a polynucleotide construct (i.e., not packaged into virus), 0.1 μg to 100 μg or more can be administered. Tumor sizes are measured and, in some experiments, blood samples are taken weekly. The effect of intratumoral injection of the adenoviral vector (such as CN733) on tumor size and serum CEA levels is compared to sham treatment.

While it is highly possible that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to determine if intravenous (IV) administration of adenovirus vector can affect tumor growth. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of three to five mice bearing LoVo tumors are inoculated with $10^8$ pfu of an adenoviral vector by tail vein injection, or with buffer used to carry the virus as a negative control. The effect of IV injection of the adenoviral vector on tumor size and serum CEA levels is compared to sham treatment.

## Example 4

## Testing a CEA-TRE using a reporter gene construct

A CEA-TRE can be tested in a reporter gene assay. Briefly, an adenoviral vector is constructed in which the reporter gene is under transcriptional control of the CEA-TRE. Reporter genes have been disclosed above, and can be inserted into an adenovirus and placed under control of a CEA-TRE. Methods for such construction are known in the art or are disclosed herein.

The CEA-TRE to be tested may have mutations such as deletions or insertions within binding sites known to be important in CEA-TRE activity, or base substitutions in these sites themselves.

| Cell line | | |
|---|---|---|
| 293 | 13.8 | 2.6 |
| LoVo | 97.2 | 1.9 |

As shown in Table 3, the plasmid with the positive orientation of a CEA-TRE driving expression of the luciferase reporter gene demonstrated a 7-fold increase in luciferase expression in LoVo cells over 293 cells. Furthermore, the plasmid in which a CEA-TRE is present but does not drive expression of luciferase demonstrated only basal levels of luciferase expression.

The CEA-TRE used in this experiment is identical to that used in construct CN741, which, as described above, demonstrated a 4-fold increase in specificity in the plaque assay when comparing 293 and HBL-100 cells.

Therefore, the data shown in this Example and Example 2 show that a CEA-TRE is capable of mediating transcription and control of adenoviral replication specific to cells that allow a CEA-TRE to function, such as CEA-expressing cells.

## Example 5

## Construction of an adenoviral vector containing the coding region for the adenovirus death protein (ADP) under control of a CEA-TRE

An adenovirus in which the ADP gene is under control of a CEA-TRE can be constructed as described below. ADP is encoded within the E3 region and naturally under control of the major late promoter (MLP). The gene appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

The ADP coding sequence from Ad2 can introduced into Ad5 in the E3 region (which is often deleted in the constructs; see Example 1), as follows.

necessary for viral replication, ORF 6, was reintroduced by PCR amplifying the ORF with primers,

33.81.1 (Ad5 33096):

GCAGCTCACTTAAGTTCATGTCG (SEQ ID NO:17)

33.81.2 (Ad5 34084):

TCAGCCTAGGAAATATGACTACGTCCG (SEQ ID NO:18)

The resulting plasmid is CN203. CN203 was digested with EcoRI and ligated to CN209, a shuttle plasmid, to generate CN208. In the final cloning step, CN208 was digested with AscI and AvrII and ligated to similarly cut E4 deletion/substitution with the ADP cassette.

Thus, both CN252 and CN270 are adenoviral derivatives containing the ADP and lacking the E3 gene. In addition, CN270 lacks some sequence in the E4 region as previously described. Full-length adenoviral vectors are obtained via *in vitro* ligation of (1) appropriately prepared viral DNA digested with BamHI and (2) CN252 or CN257 also digested with BamHI. The ligation product is used to transfect 293 cells. Plaque assays are performed as described above.

CN252 and CN270 can also be modified by insertion of a CEA-TRE fragment to place the ADP gene under control of CEA-TRE.

## Example 6

## Characterization of an E3 deleted adenovirus, CN751, that contains the adenovirus death protein gene

An adenovirus comprising an adenovirus death protein, CN751, was constructed to test whether such a construct may be more effective for cytotoxicity. The adenovirus death protein (ADP), an 11.6-kDa Asn-glycosylated integral membrane peptide expressed at high levels late in infection, migrates to the nuclear membrane of infected cells and affects efficient lysis of the host. The Adenovirus 5 (Ad5) E3 region expresses the *adp* gene.

***Construction of CN751***

detected by a simple protocol. CN751's ability to cause cell death was compared to that of CN702, a vector lacking the ADP gene, and Rec700, a vector containing the ADP gene.

Monolayers of LNCaP cells were infected at an MOI of one with either CN702, Rec700 (*adp*+ control), or CN751 and then seeded in 96 well dishes. Samples were harvested once a day from one day after infection to five days after infection and scored using Promega's Cytotox 96 kit. This assay uses a coupled enzymatic reaction which converts a tetrazolium salt to a red formazan product that can be determined in a plate reader at 490nm.

Since the absorbance of a sample corresponds to the level of LDH released from infected cells, a plot of how a sample's absorbance changes with time describes how efficiently the viruses studied induce cell lysis (Figure 6). Each data point represents the average of sixteen separate samples. The results suggest that CN751 kills cells more efficiently than the *adp*- control, CN702, and similarly to the *adp*+ control, Rec700. The concentration of LDH in the supernatant increases rapidly from two days and reaches a maximum at four days in wells infected with CN751. In contrast, LDH concentration in the supernatant of CN702 infected cells begins to rise slowly at two days and continues until the conclusion of the experiment. Significantly, the amount of LDH released from CN751 infected cells at three days is two times that released from CN702 infected cells. In sum, the virus yield data demonstrate that adenoviral vectors with the ADP gene release more virus.

Not only is it important for Ad vectors to kill cells efficiently, they must also be able to shed progeny that can infect other cancer cells. Viral vectors that can shed large amounts of virus might be better therapeutics than those that shed only small amounts. A virus yield assay was undertaken to evaluate whether CN751 can induce the efficient release of its progeny from the infected cell. A549 cells were infected at an MOI of five. Supernatant was harvested at various times after infection and titered on 293 cells to determine the virus yield (Figure 7). The data suggest that cells infected with CN751 shed virus more efficiently than those infected with CN702. At forty-eight hours post infection, CN751 infected cells released ten times more virus than CN702 infected. At seventy-two hours post infection, CN751 infected cells released forty times more virus. The data demonstrate that adenoviral vectors with the ADP gene kill cells more efficiently than adenoviral vectors that lack the ADP gene.

running a 0 to 1.5 M NaCl gradient in 50 mM tris, pH 8.0. The gradient was run over 10 column volumes.

*Characterization of PEG-CN706*

Pegylation of CN706 was verified by SDS-Page. Figure 10 depicts the pegylation of CN706 and the mobility shift of pegylated proteins. Lanes 1 and 2 are non-pegylated CN706 (control), lanes 3 through 6 are pegylated CN706 under several pH and temperature conditions. Lanes 3 through 6 show the appearance of a second band above the hexon proteins of CN706, most likely pegylated hexon, and the loss of the fiber protein band. Since no additional bands associated with the virus except that corresponding to the PEG-hexon protein, the pegylated fiber protein is assumed to be under one of the unpegylated proteins on the SDS gel.

Figure 11 is an ion exchange chromatogram showing the change in surface properties of CN706. Pegylation of CN706 results in its earlier elution from the Q Sepharose resin used to capture the virus. This result is most likely due to PEG rendering the virus more charge neutral in appearance and hence decreasing its binding potential to the ion exchange matrix. A broadening of the virus' chromatogram is expected since the pegylation of CN706 occurs to different percentages.

The infectivity of pegylated CN706 was evaluated in an *in vitro* plaque assay on 293 cells. Table 4 depicts a 5 to 10-fold reduction in plaquing efficiency of PEG-CN706 as compared to CN706. This is most likely due to pegylation masking the virus cells, decreasing the recognition and endocytosis of the viral particles.

Table 4: Comparison of Plaquing Efficiency of CN706 and PEG-CN706.

| **Sample Description** | **Number of Plaques (Arbitrary Units)** |
|---|---|
| CN706 | $15 \pm 5$ |
| PEG-CN706 | $4 \pm 1$ |

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

GGCATCATCC CACCTTCCCA GAGCCCTGGA GAGCATGGGG AGACCCGGGA CCCTGCTGGG
120

TTTCTCTGTC ACAAAGGAAA ATAATCCCCC TGGTGTGACA GACCCAAGGA CAGAACACAG
180

CAGAGGTCAG CACTGGGGAA GACAGGTTGT CCTCCCAGGG GATGGGGGTC CATCCACCTT
240

GCCGAAAAGA TTTGTCTGAG GAACTGAAAA TAGAAGGGAA AAAAGAGGAG GGACAAAAGA
300

GGCAGAAATG AGAGGGGAGG GGACAGAGGA CACCTGAATA AAGACCACAC CCATGACCCA
360

CGTGATGCTG AGAAGTACTC CTGCCCTAGG AAGAGACTCA GGGCAGAGGG AGGAAGGACA
420

GCAGACCAGA CAGTCACAGC AGCCTTGACA AAACGTTCCT GGAACTCAAG CA
472

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGTCTTCAA GAATTCTCA
19

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTCAGTCAC CGGTGTCGGA
20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCAGAAAATC CAGCAGGTAC C
21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTACCGGTA GCCACCACCC AGTGAG
26

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TAGACCGGTG CTTGAGTTCC AGGAAC
26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAACGGCCGA GCCACCACCC A
21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCAGCTCACT TAAGTTCATG TCG
23

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCAGCCTAGG AAATATGACT ACGTCCG
27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 307 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..304

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
G ATG ACC GGC TCA ACC ATC GCG CCC ACA ACG GAC TAT CGC AAC ACC
46
  Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr
   1               5                  10                  15

ACT GCT ACC GGA CTA ACA TCT GCC CTA AAT TTA CCC CAA GTT CAT GCC
94
Thr Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala
            20                  25                  30

TTT GTC AAT GAC TGG GCG AGC TTG GAC ATG TGG TGG TTT TCC ATA GCG
142
Phe Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala
        35                  40                  45
```

# CLAIMS

What is claimed is:

1. An adenovirus vector comprising an adenovirus gene under transcriptional control of a carcinoembryonic antigen transcriptional regulatory element (CEA-TRE).

2. The adenovirus vector of claim 1, wherein the adenovirus gene is essential for viral replication.

3. The adenovirus vector of claim 2, wherein the adenovirus gene is an early gene.

4. The adenovirus of claim 2, wherein the adenovirus gene is a late gene.

5. The adenovirus vector of claim 3, wherein the adenovirus early gene is E1A.

6. The adenovirus vector of claim 3, wherein the adenovirus early gene is E1B.

7. The adenovirus vector of claim 1, wherein the adenovirus gene is the adenovirus death protein gene (ADP).

8. The adenovirus vector of claim 1, wherein the CEA-TRE comprises an enhancer from a carcinoembryonic antigen gene.

9. The adenovirus vector of claim 1, wherein the CEA-TRE comprises a promoter from a carcinoembryonic antigen gene.

21. The vector of claim 19, wherein the heterologous gene is conditionally required for cell survival.

22. A host cell transformed with an adenovirus vector of claim 1.

23. A host cell transformed with an adenovirus vector of claim 16.

24. A method of detecting cells that allow a CEA-TRE to function in a biological sample comprising the steps of:

contacting a biological sample with an adenovirus vector of claim 1, under conditions suitable for CEA-TRE-mediated gene expression in cells that allow a CEA-TRE to function; and

determining if CEA-TRE mediates gene expression in the biological sample,

wherein CEA-TRE-mediated gene expression is indicative of the presence of cells that allow a CEA-TRE to function.

25. A method of propagating adenovirus specific for cells that allow a CEA-TRE to function, said method comprising:

combining an adenovirus according to claim 1 with cells that allow a CEA-TRE to function,

whereby said adenovirus is propagated.

26. A method of propagating an adenovirus specific for cells that allow a CEA-TRE to function, said method comprising:

combining an adenovirus according to claim 16 with cells that allow a CEA-TRE to function,

36. The adenovirus of claim 35, wherein the PEG is of a molecular weight between about 3000 to about 20,000.

37. The adenovirus of claim 36, wherein the PEG is of a molecular weight between about 5000 to about 10,000.

38. The adenovirus of claim 34, wherein the PEG is covalently attached to the adenovirus.

39. The adenovirus of claim 34, wherein the PEG is non-covalently attached to the adenovirus.

40. The adenovirus of claim 38, wherein the PEG is covalently attached by using a N-hydroxysuccinimidyl (NHS) active ester.

41. The adenovirus of claim 40, wherein the N-hydroxysuccinimidyl (NHS) active ester is selected from the group consisting of succinimidyl succinate, succinimidyl succinamide and succinimidyl propionate.

42. The adenovirus of claim 41, wherein the N-hydroxysuccinimidyl (NHS) active ester is succinimidyl succinate.

43. A method of making a masked adenovirus , comprising covalently attaching a masking agent to an adenovirus, wherein the masking agent is has a molecular weight between about 2500 and about 20,000, thereby producing a masked adenovirus.

44. The method of claim 44, wherein the masking agent is polyethyleneglycol (PEG).

45. An adenovirus complexed with a masking agent.

46. The adenovirus of claim 45 wherein the masking agent is polyethyleneglycol (PEG).

47. The adenovirus of claim 46, wherein the PEG is of a molecular weight between about 2500 to about 30,000.

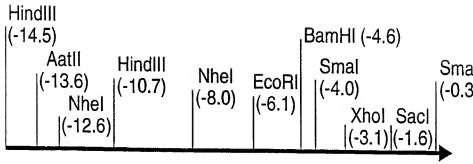
HindIII
(-14.5)
AatII
(-13.6)
NheI
(-12.6)
HindIII
(-10.7)
NheI
(-8.0)
EcoRI
(-6.1)
BamHI (-4.6)
SmaI
(-4.0)
XhoI
(-3.1)
SacI
(-1.6)
SmaI
(-0.3)

FIG. 1

-13023 CCATTTGGCA AGAGACATAA AGGACATTCC AGGACATGCC TTCCTGGGAG GTCCAGGTTC

-12963 TCTGTCTCAC ACCTCAGGGA CTGTAGTTAC TGCATCAGCC ATGGTAGGTG CTGATCTCAC

-12903 CCAGCCTGTC CAGGCCCTTC CACTCTCCAC TTTGTGACCA TGTCCAGGAC CACCCCTCAG

-12843 ATCCTGAGCC TGCAAATACC CCCTTGCTGG GTGGGTGGAT TCAGTAAACA GTGAGCTCCT

-12783 ATCCAGCCCC CAGAGCCACC TCTGTCACCT TCCTGCTGGG CATCATCCCA CCTTCACAAG

-12723 CACTAAAGAG CATGGGGAGA CCTGGCTAGC TGGGTTTCTG CATCACAAAG AAAATAATCC

-12663 CCCAGGTTCG GATTCCCAGG GCTCTGTATG TGGAGCTGAC AGACCTGAGG CCAGGAGATA

-12603 GCAGAGGTCA GCCCTAGGGA GGGTGGGTCA TCCACCCAGG GGACAGGGGT GCACCAGCCT

-12543 TGCTACTGAA AGGGCCTCCC CAGGACAGCG CCATCAGCCC TGCCTGAGAG CTTTGCTAAA

-12483 CAGCAGTCAG AGGAGGCCAT GGCAGTGGCT GAGCTCCTGC TCCAGGCCCC AACAGACCAG

-12423 ACCAACAGCA CAATGCAGTC CTTCCCCAAC GTCACAGGTC ACCAAAGGGA AACTGAGGTG

-12363 CTACCTAACC TTAGAGCCAT CAGGGGAGAT AACAGCCCAA TTTCCCAAAC AGGCCAGTTT

-12303 CAATCCCATG ACAATGACCT CTCTGCTCTC ATTCTTCCCA AAATAGGACG CTGATTCTCC

-12243 CCCACCATGG ATTTCTCCCT TGTCCCGGGA GCCTTTTCTG CCCCCTATGA TCTGGGCACT

-12183 CCTGACACAC ACCTCCTCTC TGGTGACATA TCAGGGTCCC TCACTGTCAA GCAGTCCAGA

-12123 AAGGACAGAA CCTTGGACAG CGCCCATCTC AGCTTCACCC TTCCTCCTTC ACAGGGTTCA

-12063 GGGCAAAGAA TAAATGGCAG AGGCCAGTGA GCCCAGAGAT GGTGACAGGC AGTGACCCAG

-12003 GGGCAGATGC CTGGAGCAGG AGCTGGCGGG GCCACAGGGA GAAGGTGATG CAGGAAGGGA

-11943 AACCCAGAAA TGGGCAGGAA AGGAGGACAC AGGCTCTGTG GGGCTGCAGC CCAGGGTTGG

-11883 ACTATGAGTG TGAAGCCATC TCAGCAAGTA AGGCCAGGTC CCATGAACAA GAGTGGGAGC

-11823 ACGTGGCTTC CTGCTCTGTA TATGGGGTGG GGGATTCCAT GCCCCATAGA ACCAGATGGC

-11763 CGGGGTTCAG ATGGAGAAGG AGCAGGACAG GGGATCCCCA GGATAGGAGG ACCCCAGTGT

-11703 CCCCACCCAG GCAGGTGACT GATGAATGGG CATGCAGGGT CCTCCTGGGC TGGGCTCTCC

-11643 CTTTGTCCCT CAGGATTCCT TGAAGGAACA TCCGGAAGCC GACCACATCT ACCTGGTGGG

FIG. 2B

-10143 ACGCCTGTAA TCCCAGCACT TTAGAAGGCT GAGGTGGGCA GATTACTTGA GGTCAGGAGT

-10083 TCAAGACCAC CCTGGCCAAT ATGGTGAAAC CCCGGCTCTA CTAAAAATAC AAAAATTAGC

-10023 TGGGCATGGT GGTGCGCGCC TGTAATCCCA GCTACTCGGG AGGCTGAGGC TGGACAATTG

-9963 CTTGGACCCA GGAAGCAGAG GTTGCAGTGA GCCAAGATTG TGCCACTGCA CTCCAGCTTG

-9903 GGCAACAGAG CCAGACTCTG TAAAAAAAAA AAAAAAAAAA AAAAAAAGAA AGAAAGAAAA

-9843 AGAAAAGAAA GTATAAAATC TCTTTGGGTT AACAAAAAAA GATCCACAAA ACAAACACCA

-9783 GCTCTTATCA AACTTACACA ACTCTGCCAG AGAACAGGAA ACACAAATAC TCATTAACTC

-9723 ACTTTTGTGG CAATAAAACC TTCATGTCAA AAGGAGACCA GGACACAATG AGGAAGTAAA

-9663 ACTGCAGGCC CTACTTGGGT GCAGAGAGGG AAAATCCACA AATAAAACAT TACCAGAAGG

-9603 AGCTAAGATT TACTGCATTG AGTTCATTCC CCAGGTATGC AAGGTGATTT TAACACCTGA

-9543 AAATCAATCA TTGCCTTTAC TACATAGACA GATTAGCTAG AAAAAAATTA CAACTAGCAG

-9483 AACAGAAGCA ATTTGGCCTT CCTAAAATTC CACATCATAT CATCATGATG GAGACAGTGC

-9423 AGACGCCAAT GACAATAAAA AGAGGGACCT CCGTCACCCG GTAAACATGT CCACACAGCT

-9363 CCAGCAAGCA CCCGTCTTCC CAGTGAATCA CTGTAACCTC CCCTTTAATC AGCCCCAGGC

-9303 AAGGCTGCCT GCGATGGCCA CACAGGCTCC AACCCGTGGG CCTCAACCTC CCGCAGAGGC

-9243 TCTCCTTTGG CCACCCCATG GGGAGAGCAT GAGGACAGGG CAGAGCCCTC TGATGCCCAC

-9183 ACATGGCAGG AGCTGACGCC AGAGCCATGG GGGCTGGAGA GCAGAGCTGC TGGGGTCAGA

-9123 GCTTCCTGAG GACACCCAGG CCTAAGGGAA GGCAGCTCCC TGGATGGGGG CAACCAGGCT

-9063 CCGGGCTCCA ACCTCAGAGC CCGCATGGGA GGAGCCAGCA CTCTAGGCCT TTCCTAGGGT

-9003 GACTCTGAGG GGACCCTGAC ACGACAGGAT CGCTGAATGC ACCCGAGATG AAGGGGCCAC

-8943 CACGGGACCC TGCTCTCGTG GCAGATCAGG AGAGAGTGGG ACACCATGCC AGGCCCCCAT

-8883 GGCATGGCTG CGACTGACCC AGGCCACTCC CCTGCATGCA TCAGCCTCGG TAAGTCACAT

-8823 GACCAAGCCC AGGACCAATG TGGAAGGAAG GAAACAGCAT CCCCTTTAGT GATGGAACCC

-8763 AAGGTCAGTG CAAAGAGAGG CCATGAGCAG TTAGGAAGGG TGGTCCAACC TACAGCACAA

FIG. 2D

-7263 TTGGCCTGGA GGCCACTGGT CCCCTCTGTC CCTGAGGGGA ATCTGCACCC TGGAGGCTGC

-7203 CACATCCCTC CTGATTCTTT CAGCTGAGGG CCCTTCTTGA AATCCCAGGG AGGACTCAAC

-7143 CCCCACTGGG AAAGGCCCAG TGTGGACGGT TCCACAGCAG CCCAGCTAAG GCCCTTGGAC

-7083 ACAGATCCTG AGTGAGAGAA CCTTTAGGGA CACAGGTGCA CGGCCATGTC CCCAGTGCCC

-7023 ACACAGAGCA GGGGCATCTG GACCCTGAGT GTGTAGCTCC CGCGACTGAA CCCAGCCCTT

-6963 CCCCAATGAC GTGACCCCTG GGGTGGCTCC AGGTCTCCAG TCCATGCCAC CAAAATCTCC

-6903 AGATTGAGGG TCCTCCCTTG AGTCCCTGAT GCCTGTCCAG GAGCTGCCCC CTGAGCAAAT

-6843 CTAGAGTGCA GAGGGCTGGG ATTGTGGCAG TAAAAGCAGC CACATTTGTC TCAGGAAGGA

-6783 AAGGGAGGAC ATGAGCTCCA GGAAGGGCGA TGGCGTCCTC TAGTGGGCGC CTCCTGTTAA

-6723 TGAGCAAAAA GGGGCCAGGA GAGTTGAGAG ATCAGGGCTG GCCTTGGACT AAGGCTCAGA

-6663 TGGAGAGGAC TGAGGTGCAA AGAGGGGGCT GAAGTAGGGG AGTGGTCGGG AGAGATGGGA

-6603 GGAGCAGGTA AGGGGAAGCC CCAGGGAGGC CGGGGGAGGG TACAGCAGAG CTCTCCACTC

-6543 CTCAGCATTG ACATTTGGGG TGGTCGTGCT AGTGGGGTTC TGTAAGTTGT AGGGTGTTCA

-6483 GCACCATCTG GGGACTCTAC CCACTAAATG CCAGCAGGAC TCCCTCCCCA AGCTCTAACA

-6423 ACCAACAATG TCTCCAGACT TTCCAAATGT CCCCTGGAGA GCAAAATTGC TTCTGGCAGA

-6363 ATCACTGATC TACGTCAGTC TCTAAAAGTG ACTCATCAGC GAAATCCTTC ACCTCTTGGG

-6303 AGAAGAATCA CAAGTGTGAG AGGGGTAGAA ACTGCAGACT TCAAAATCTT TCCAAAAGAG

-6243 TTTTACTTAA TCAGCAGTTT GATGTCCCAG GAGAAGATAC ATTTAGAGTG TTTAGAGTTG

-6183 ATGCCACATG GCTGCCTGTA CCTCACAGCA GGAGCAGAGT GGGTTTTCCA AGGGCCTGTA

-6123 ACCACAACTG GAATGACACT CACTGGGTTA CATTACAAAG TGGAATGTGG GGAATTCTGT

-6063 AGACTTTGGG AAGGGAAATG TATGACGTGA GCCCACAGCC TAAGGCAGTG GACAGTCCAC

-6003 TTTGAGGCTC TCACCATCTA GGAGACATCT CAGCCATGAA CATAGCCACA TCTGTCATTA

-5943 GAAAACATGT TTTATTAAGA GGAAAAATCT AGGCTAGAAG TGCTTTATGC TCTTTTTTCT

-5883 CTTTATGTTC AAATTCATAT ACTTTTAGAT CATTCCTTAA AGAAGAATCT ATCCCCCTAA

FIG. 2F

-4383 AGACCCCTGC ACCAATGAGA CCATGCTGAA GCCTCAGAGA GAGAGATGGA GCTTTGACCA

-4323 GGAGCCGCTC TTCCTTGAGG GCCAGGGCAG GGAAAGCAGG AGGCAGCACC AGGAGTGGGA

-4263 ACACCAGTGT CTAAGCCCCT GATGAGAACA GGGTGGTCTC TCCCATATGC CCATACCAGG

-4203 CCTGTGAACA GAATCCTCCT TCTGCAGTGA CAATGTCTGA GAGGACGACA TGTTTCCCAG

-4143 CCTAACGTGC AGCCATGCCC ATCTACCCAC TGCCTACTGC AGGACAGCAC CAACCCAGGA

-4083 GCTGGGAAGC TGGGAGAAGA CATGGAATAC CCATGGCTTC TCACCTTCCT CCAGTCCAGT

-4023 GGGCACCATT TATGCCTAGG ACACCCACCT GCCGGCCCCA GGCTCTTAAG AGTTAGGTCA

-3963 CCTAGGTGCC TCTGGGAGGC CGAGGCAGGA GAATTGCTTG AACCCGGGAG GCAGAGGTTG

-3903 CAGTGAGCCG AGATCACACC ACTGCACTCC AGCCTGGGTG ACAGAATGAG ACTCTGTCTC

-3843 AAAAAAAAAG AGAAAGATAG CATCAGTGGC TACCAAGGGC TAGGGGCAGG GGAAGGTGGA

-3783 GAGTTAATGA TTAATAGTAT GAAGTTTCTA TGTGAGATGA TGAAAATGTT CTGGAAAAAA

-3723 AAATATAGTG GTGAGGATGT AGAATATTGT GAATATAATT AACGGCATTT AATTGTACAC

-3663 TTAACATGAT TAATGTGGCA TATTTTATCT TATGTATTTG ACTACATCCA AGAAACACTG

-3603 GGAGAGGGAA AGCCCACCAT GTAAAATACA CCCACCCTAA TCAGATAGTC CTCATTGTAC

-3543 CCAGGTACAG GCCCCTCATG ACCTGCACAG GAATAACTAA GGATTTAAGG ACATGAGGCT

-3483 TCCCAGCCAA CTGCAGGTGC ACAACATAAA TGTATCTGCA AACAGACTGA GAGTAAAGCT

-3423 GGGGGCACAA ACCTCAGCAC TGCCAGGACA CACACCCTTC TCGTGGATTC TGACTTTATC

-3363 TGACCCGGCC CACTGTCCAG ATCTTGTTGT GGGATTGGGA CAAGGGAGGT CATAAAGCCT

-3303 GTCCCCAGGG CACTCTGTGT GAGCACACGA GACCTCCCCA CCCCCCCACC GTTAGGTCTC

-3243 CACACATAGA TCTGACCATT AGGCATTGTG AGGAGGACTC TAGCGCGGGC TCAGGGATCA

-3183 CACCAGAGAA TCAGGTACAG AGAGGAAGAC GGGGCTCGAG GAGCTGATGG ATGACACAGA

-3123 GCAGGGTTCC TGCAGTCCAC AGGTCCAGCT CACCCTGGTG TAGGTGCCCC ATCCCCCTGA

-3063 TCCAGGCATC CCTGACACAG CTCCCTCCCG GAGCCTCCTC CCAGGTGACA CATCAGGGTC

-3003 CCTCACTCAA GCTGTCCAGA GAGGGCAGCA CCTTGGACAG CGCCCACCCC ACTTCACTCT

FIG. 2H

-1503 AGGGCAGCTC CCTGTGATCT CCAGGACAGC TCAGTCTCTC ACAGGCTCCG ACGCCCCCTA

-1443 TGCTGTCACC TCACAGCCCT GTCATTACCA TTAACTCCTC AGTCCCATGA AGTTCACTGA

-1383 GCGCCTGTCT CCCGGTTACA GGAAAACTCT GTGACAGGGA CCACGTCTGT CCTGCTCTCT

-1323 GTGGAATCCC AGGGCCCAGC CCAGTGCCTG ACACGGAACA GATGCTCCAT AAATACTGGT

-1263 TAAATGTGTG GGAGATCTCT AAAAAGAAGC ATATCACCTC CGTGTGGCCC CCAGCAGTCA

-1203 GAGTCTGTTC CATGTGGACA CAGGGGCACT GGCACCAGCA TGGGAGGAGG CCAGCAAGTG

-1143 CCCGCGGCTG CCCCAGGAAT GAGGCCTCAA CCCCCAGAGC TTCAGAAGGG AGGACAGAGG

-1083 CCTGCAGGGA ATAGATCCTC CGGCCTGACC CTGCAGCCTA ATCCAGAGTT CAGGGTCAGC

-1023 TCACACCACG TCGACCCTGG TCAGCATCCC TAGGGCAGTT CCAGACAAGG CCGGAGGTCT

-963 CCTCTTGCCC TCCAGGGGGT GACATTGCAC ACAGACATCA CTCAGGAAAC GGATTCCCCT

-903 GGACAGGAAC CTGGCTTTGC TAAGGAAGTG GAGGTGGAGC CTGGTTTCCA TCCCTTGCTC

-843 CAACAGACCC TTCTGATCTC TCCCACATAC CTGCTCTGTT CCTTTCTGGG TCCTATGAGG

-783 ACCCTGTTCT GCCAGGGGTC CCTGTGCAAC TCCAGACTCC CTCCTGGTAC CACCATGGGG

-723 AAGGTGGGGT GATCACAGGA CAGTCAGCCT CGCAGAGACA GAGACCACCC AGGACTGTCA

-663 GGGAGAACAT GGACAGGCCC TGAGCCGCAG CTCAGCCAAC AGACACGGAG AGGGAGGGTC

-603 CCCCTGGAGC CTTCCCCAAG GACAGCAGAG CCCAGAGTCA CCCACCTCCC TCCACCACAG

-543 TCCTCTCTTT CCAGGACACA CAAGACACCT CCCCCTCCAC ATGCAGGATC TGGGGACTCC

-483 TGAGACCTCT GGGCCTGGGT CTCCATCCCT GGGTCAGTGG CGGGGTTGGT GGTACTGGAG

-423 ACAGAGGGCT GGTCCCTCCC CAGCCACCAC CCAGTGAGCC TTTTTCTAGC CCCCAGAGCC

-363 ACCTCTGTCA CCTTCCTGTT GGGCATCATC CCACCTTCCC AGAGCCCTGG AGAGCATGGG

-303 GAGACCCGGG ACCCTGCTGG GTTTCTCTGT CACAAAGGAA AATAATCCCC CTGGTGTGAC

-243 AGACCCAAGG ACAGAACACA GCAGAGGTCA GCACTGGGGA AGACAGGTTG TCCTCCCAGG

-183 GGATGGGGGT CCATCCACCT TGCCGAAAAG ATTTGTCTGA GGAACTGAAA ATAGAAGGGA

-123 AAAAAGAGGA GGGACAAAAG AGGCAGAAAT GAGAGGGGAG GGGACAGAGG ACACCTGAAT

FIG. 2J

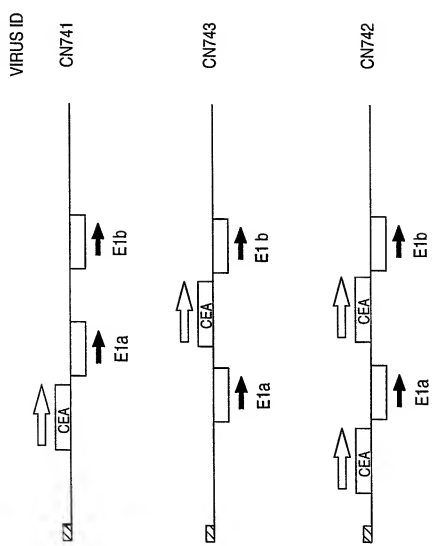
VIRUS ID
CN741
CN743
CN742
PLASMID ID#
CN266
CN290
CN285
CEA
E1a
E1b

FIG. 3

E3 mRNA CAP SITE
Y LEADER
ADENOVIRUS DEATH PROTEIN CODING REGION
E3A POLY A SITE
E3B POLY A SITE
27609bp
28375bp
28559bp
29468bp
29773bp
29792bp
31078bp
37.124.1
28287bp
37.146.1
28622bp
37.124.3
29195bp
37.124.4
29872bp

FIG. 5A


28287bp
28622bp
29195bp
29872bp

FIG. 5B

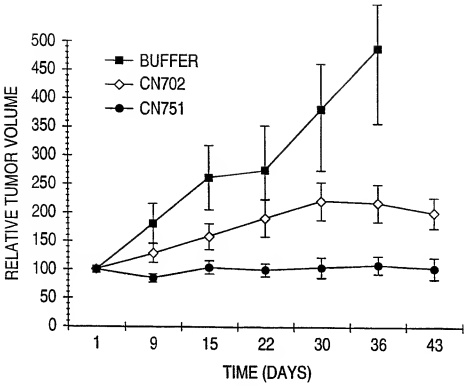
BUFFER
CN702
CN751
RELATIVE TUMOR VOLUME
500
450
400
350
300
250
200
150
100
50
0
1
9
15
22
30
36
43
TIME (DAYS)

FIG. 8

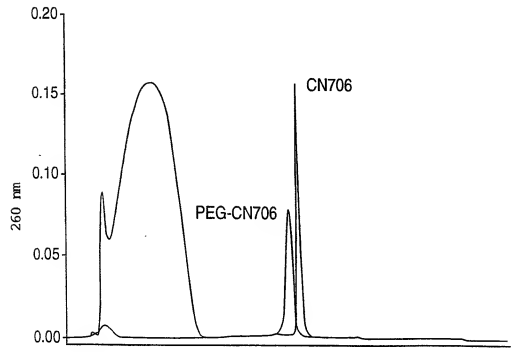
0.20
0.15
0.10
0.05
0.00
260 nm
CN706
PEG-CN706

FIG. 11

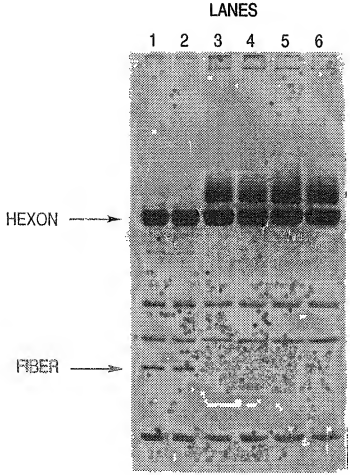


FIG. 10

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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A3

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(72) Inventors; and

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(54) Title: ADENOVIRUS VECTORS SPECIFIC FOR CELLS EXPRESSING CARCINOEMBRYONIC ANTIGEN AND METHODS OF USE THEREOF

(57) Abstract

Replication-competent adenovirus vectors specific for cells expressing carcinoembryonic antigen (CEA), and methods of use of such viruses are provided. These viruses comprise an adenoviral gene under control of a CEA transcriptional regulatory element (CEA–TRE). The gene can be, for example, a gene required for viral replication or the adenovirus death protein gene (ADP). The viruses can also comprise at least one other adenoviral gene under control of another transcriptional regulatory element specific to cells capable of which allow a CEA–TRE to function, such as a variant of CEA–TRE. By providing for transcriptional initiating regulation dependent upon CEA expression, virus replication can be restricted to target cells which allow a CEA–TRE to function, such as cells expressing CEA, particularly carcinoma cells capable of expressing CEA. An adenovirus of the present invention can further comprise a heterologous gene such as a reporter gene under transcriptional control of a CEA–TRE. The adenovirus vectors can be used to detect and monitor samples for the presence of cells that allow a CEA–TRE to function, as well as to selectively kill malignant cells that allow a CEA–TRE to function.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 A61K48/00 A61K47/48 C12Q1/70
C12N11/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 96 17053 A (GENETIC THERAPY INC ;HALLENBECK PAUL L (US); CHANG YUNG NIEN (US);) 6 June 1996 cited in the application see the whole document --- | 1-32 |
| X | WO 96 34969 A (CANJI INC) 7 November 1996 see the whole document, especially page 17, lines 9 to 30 --- | 1-32 |
| Y | WO 95 14100 A (WELLCOME FOUND ;RICHARDS CYNTHIA ANN (US); HUBER BRIAN (US)) 26 May 1995 cited in the application see the whole document --- | 1-32 |
| Y | WO 97 01358 A (CALYDON) 16 January 1997 see the whole document --- -/-- | 1-32 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 10 July 1998 | 26. 10. 98 |

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Authorized officer
Mandl, B

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/04133

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 27-30, as far as an in vivo application is concerned, and claims 31 and 32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see further information sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-42

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9617053 A | 06-06-96 | AU 4504396 A | 19-06-96 |
| | | CA 2206179 A | 06-06-96 |
| | | EP 0791050 A | 27-08-97 |
| | | FI 972225 A | 16-07-97 |
| | | NO 972373 A | 25-07-97 |
| WO 9634969 A | 07-11-96 | AU 5723696 A | 21-11-96 |
| | | CA 2218390 A | 07-11-96 |
| | | EP 0827546 A | 11-03-98 |
| WO 9514100 A | 26-05-95 | AU 1071295 A | 06-06-95 |
| | | BR 9408101 A | 05-08-97 |
| | | CA 2176014 A | 26-05-95 |
| | | EP 0729515 A | 04-09-96 |
| | | FI 962104 A | 17-05-96 |
| | | HU 75235 A | 28-04-97 |
| | | JP 9504957 T | 20-05-97 |
| | | NZ 276447 A | 27-04-98 |
| | | ZA 9409197 A | 20-05-96 |
| WO 9701358 A | 16-01-97 | US 5698443 A | 16-12-97 |
| | | AU 6393296 A | 30-01-97 |
| | | CA 2222457 A | 16-01-97 |
| | | EP 0844888 A | 03-06-98 |